THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

III. The Malignant Fibroblast of Sarcoma 10 of the Crocker Foundation.

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Plate 4.

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The definition of a cell by its morphological characteristics, according to classical cytology, is of an unsatisfactory nature because the individuality of an anatomical element depends more on its physiological properties than on its appearance.[1] A description of a given structure remains almost without significance if the relations that correlate its form and function remain unknown. Such a study should always be completed by a thorough investigation of the fundamental physiological properties of the element considered. In this manner, the individuality of the fibroblast and the macrophage has become clearly defined;[2,3] but an investigation of the sort must be undertaken for every cell type that has been obtained in pure culture. If a similar study were made of the cells composing experimental tumors, the nature of malignancy might soon be discovered. A search has already been started for the essential characteristics of the specific elements of Rous sarcoma,[4] of rat sarcomas,[5] and of mouse and rat carcinomas,[6] with the object of ascertaining the factors which cause

[1] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1198. Carrel, A., in Cowdry, E. V., Special cytology, New York, 1928, 1.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 261.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 285.

[4] Carrel, A., *Compt. rend. Soc. biol.*, 1925, xcii, 584.

[5] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1119.

[6] Fischer, A., *Z. Krebsforsch.*, 1927, xxv, 89. Laser, H., *Z. Krebsforsch.*, 1927, xxv, 298.

these cells to multiply indefinitely within the organism. The purpose of the present paper is to describe the properties that distinguish the malignant fibroblast of Sarcoma 10 of the Crocker Foundation from the normal fibroblast of the rat, when both cell types are living in pure cultures.

Isolation of a Strain of Malignant Fibroblasts.

Rat Sarcoma 10, which was obtained by us from the Crocker Foundation through the kindness of Dr. F. C. Wood, is a tumor easily transmissible by transplantation. Its growth is rapid. It spreads locally, reaches a very large size, and kills the animal by cachexia. There are no metastases. Regression of the tumor is exceptional. It is composed of large, short spindle cells with oval nuclei and one or two nucleoli, densely packed together. Between the cells are scattered many macrophages which may easily be distinguished from the fibroblasts by the appearance of their nucleus. When a pure culture of malignant fibroblasts is inoculated into a rat, the tumor which develops after less than 5 days already shows many macrophages mixed with the spindle cells. The macrophages thus appear to be a normal constituent of the tumor.

In November, 1926, a few small fragments of Sarcoma 10 were cultivated in D flasks containing a solid medium composed of chicken plasma. When chicken plasma is diluted with 3 volumes of Tyrode solution, coagulated with 1 drop of chick embryo juice, and washed once or several times in an excess of Tyrode solution, it yields a coagulum which is not toxic for foreign cells.[7] This coagulum has the advantage of remaining transparent and of not being digested by the rat tissues.

Some diluted chick embryo juice was injected at the surface of the coagulum as a nutrient medium, for it is known that rabbit, guinea pig, rat, fowl, and other animal tissues, utilize foreign embryo proteins for the building up of new protoplasm.[8] During the first 24 hours of incubation, the tissue fragments surrounded themselves with a crown of macrophages. The migration of these macrophages was more abundant in a nutrient medium composed of heparinized rat plasma or of rat serum, than when embryonic juice was used. In rat serum or heparinized plasma, the macrophages spread rapidly

[7] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 601.

[8] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 603. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 499.

throughout the medium. But in a nutrient medium composed exclusively of chick embryo juice, they grew very much less extensively, while the fibroblasts rapidly increased in number. Every 2 or 3 days, after the cultures had been washed in Tyrode solution, the nutrient medium was changed. When the tissues had grown for about 10 days, the solid medium was removed from the flask, spread on a glass plate, and the area of growth isolated by four sharp cuts of a cataract knife. It was then divided into two parts and placed in a fresh flask. After a few passages in embryo tissue juice, all the macrophages disappeared, and a pure strain of large, short fibroblasts, similar to those seen in the sections of the tumor, was obtained. The morphology of the cells varied considerably according to the medium. They often appeared as long and densely packed fibroblasts which invaded the solid medium as a thick tissue. The rate of growth was measured by the ordinary technique.[9]

From time to time, a few fragments of cultures were inserted in the subcutaneous connective tissue of rats. 4 or 5 days after inoculations made in December, 1926, when the strain of fibroblasts was already free from macrophages, a small tumor appeared at the site of injection, grew rapidly, and eventually killed the animals. The fibroblasts evidently carried the malignant characteristics. Similar experiments were done later, with identical results. In January, and February, 1928, inoculations of the cultures still produced tumors within 4 or 5 days, which grew rapidly afterwards. There is, then, no doubt that malignancy is a permanent property of the strain.

A pure strain of normal fibroblasts was obtained from the rat by the ordinary technique.[9] It was cultivated under the same conditions as the sarcomatous cells, and used as a means of comparison. The morphological and physiological properties of the normal and malignant strains were studied in the same manner as those of the normal chicken fibroblasts described in a previous article.[2]

Morphological Characteristics of the Cells.

The normal and sarcomatous fibroblasts were compared after they had been cultivated for a few days in identical media, and when their

[9] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

rates of growth had become similar. Such preliminary conditions were necessary since it has been found that the nuclear and protoplasmic structures of normal fibroblasts vary in a large measure according to their metabolic state.[2] Were the functional state of the cells not ascertained, phenomena due to nutritional or degenerative changes might be falsely attributed to pathological factors.

In the following experiments, the normal and sarcomatous cells respectively were cultivated for several days in a medium composed of embryo tissue juice. Then, a few fragments of the cultures were transferred to hanging drops of plasma and embryonic juice. From 1 to 48 hours after the cover glasses were prepared, the tissues were stained with 1/20,000 Janus green and 1/50,000 neutral red. Camera lucida drawings were made of the cells at a magnification of 1,600 to 3,200 diameters.

The normal fibroblasts of the rat closely resembled the chicken fibroblasts previously described.[2] They were elongated cells with sharp boundaries and processes open at the end. The nuclei were long and oval, with one or two nucleoli. In about 0.5 per cent of the cells, two or three nuclei were observed. The dimensions of the cells averaged approximately $30 \times 115\mu$, and those of the nuclei $8.5 \times 19\mu$. The projected areas of the cell and nucleus were, respectively, 1,960 and 189 sq. μ. The segregation apparatus was small, and localized in the forward part of the cytoplasm around the centriole. Long filamentous mitochondria were seen around the nucleus and within the processes (Fig. 1, *A*).

The malignant cells were generally larger and coarser than the normal ones, and the cytoplasm was more refringent. Their length was about 125μ, and their width 39μ. The nucleus was globular, $12 \times 18.8\mu$, and wider but a little shorter than that of the normal cells. The projected areas of the cell and nucleus were, respectively, 2,300 and 230 sq. μ. The segregation apparatus was very small. There were no degenerative vacuoles or abnormal mitoses. The mitochondria were similar to those of normal cells (Fig. 1, *B*). Multinuclear cells were present in the proportion of about 0.5 per cent. Some of these cells contained a large number of small nuclei. However, the percentage of the abnormalities did not exceed that present in normal cultures. The malignant fibroblasts apparently did not de-

generate or die when cultivated as a pure strain. They looked like healthy cells distinguished from normal fibroblasts merely by their size and the particular appearance of their cytoplasm. Sarcomatous and normal fibroblasts may indeed assume, in some cultures, an identical form. So far, no morphological characteristic has been discovered which can be considered as specific of malignancy.

The cinematographic records of normal fibroblasts of the rat showed that their mode of locomotion is identical with that of chicken fibroblasts. The cells generally move out from the center of the colony in a straight line. Their activity is polarized, the outer pole being characterized by the presence of the centriole. The protoplasm streams through the apparently rigid walls of the open end of the front process. Then the nucleus and the cytoplasmic organs glide forward, dragging the rear process. Sarcomatous fibroblasts showed no essential differences, even when both normal and malignant strains were cultivated in the same medium and cinematographed simultaneously.

Architecture of the Colonies.

Normal fibroblasts never grow as isolated units. They do not scatter through the medium as macrophages do. When they migrate into the surrounding coagulum, they remain in intimate reciprocal contact on all sides and multiply actively when packed together. A fibroblast colony always forms a dense tissue. This characteristic establishes a fundamental difference between fibroblasts and macrophages. Macrophages live as independent units and die if they congregate in masses. Normal rat fibroblasts form round or oblong colonies, which are similar in appearance to those of chicken fibroblasts. They never invade the entire medium. When a small colony of rat fibroblasts is cultivated in a washed coagulum of chicken plasma in a D flask, and fed upon diluted chick embryo juice at a pH of about 7.4, it reaches a diameter of about 8 or 10 mm. in 5 or 6 days. Later, the rate of growth decreases and the cells have a tendency to degenerate. Under the present conditions, in order to keep its activity, the strain must be transferred to a fresh coagulum at the end of 6 days. The limitation in the size of the colonies is probably due to the same cause which also prevents chicken fibroblasts from invading the entire medium. In the colonies of rat fibroblasts, a thick center forms more often than is the case with

chicken fibroblasts, and degeneration begins unless the necrotic tissue is removed with a cataract knife. There is no real difference between the colonies of normal rat and chicken fibroblasts.

The colonies of sarcoma fibroblasts are similar to those of the normal type. The cells never scatter into the medium. They organize as a tissue and form round or oblong colonies. These colonies are thicker than those of normal fibroblasts and are easily recognized on account of this trait. They also reach a greater size, as they actively invade the medium for 10 or 12 days. Then, their diameter may be 15 or even 20 mm. They differ from the colonies of normal fibroblasts by their large surface and their greater opacity, and not by their architecture.

Cultures of normal and of sarcomatous fibroblasts which were placed side by side in a flask exchanged cells freely. Cinematograph records taken at the beginning of the symbiosis clearly showed that normal cells were not repelled or destroyed by the malignant ones. At this time, there was no morphological difference between the two types of cells. After a few days, composite colonies were obtained made up of normal and tumor tissues. These colonies were divided and transferred to flasks, and both types of cells, which had become easily distinguishable morphologically, were observed to migrate into the medium. But after some time, the sarcomatous tissue progressively invaded the normal tissue, which ultimately disappeared almost completely.

Residual Growth Energy.

It is well known that the inherent growth energy of a fragment of fresh tissue or of a pure culture of tissue cells can be measured by its residual energy,[10] that is, by the duration of the life of the cells and the activity they display when deprived of nitrogenous food in a medium composed of Tyrode solution. The residual energy of tissue cells probably depends on their capacity for accumulating food material while being cultivated in a nutrient medium. A comparison was made between the residual energy of colonies of normal and sarcomatous fibroblasts after they had lived for some weeks in embryonic tissue juice. Six experiments were performed, as reported in Table I.

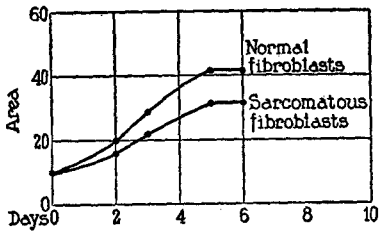
[10] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 521.

Text-fig. 1 expresses the results of an experiment which is typical. The duration of life of both normal and sarcomatous fibroblasts in Tyrode solution did not exceed 6 days. The relative increase of the normal colonies was slightly greater than that of the sarcomatous ones. The residual growth energies of both cell types were approximately equal.

TABLE I.

Effect of Tyrode Solution on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Normal fibroblasts		Sarcomatous fibroblasts	
		Duration of life	Relative increase in Tyrode solution	Duration of life	Relative increase in Tyrode solution
		days		*days*	
1	1513-H	5	0.95	5	1.40
2	1513-H	5	2.39	5	1.80
3	1517-H	9	4.30	9	1.58
4	6499-C	5	2.96	5	2.15
5	6499-C	5	3.45	5	3.07
6	6512-C	4	2.44	5	1.23
Average.....		5.5	2.75	5.67	1.87



TEXT-FIG. 1. Experiment 6499-C. Residual activity of normal and sarcomatous fibroblasts of the rat.

Duration and Rate of Growth.

When cultivated in a nutrient medium, normal rat fibroblasts multiplied in an unlimited manner, as chicken fibroblasts do. After about 16 months of life *in vitro*, sarcomatous fibroblasts proliferated as actively as at the beginning of their period of cultivation. They had also kept their malignancy. The duration of life *in vitro* of sarcomatous and normal fibroblasts appears to be unlimited.

The rate of growth of both strains has been ascertained by the measurement of the area of the colonies, and also by the volume of the new tissue which develops in a medium composed of chick embryo juice containing about 10 mg. of nitrogen per 100 cc. In Table II, the results of nine experiments are summarized, in which the rates of growth of normal and sarcomatous fibroblasts were compared for periods of 5 or 6 days. The ratio of the relative increases of the colonies in nutrient and non-nutrient media was slightly larger in the case of the normal fibroblasts. There was no fundamental difference in the rate of growth of both types of cells, as long as they were cultivated in a solution containing embryo proteins.

Effects of Normal and Sarcomatous Fibroblasts on Their Medium.

1. Liquefaction of Fibrin.—When normal and sarcomatous cells were cultivated in washed chicken plasma, no digestion of the coagulum ever occurred. The medium always remained homogeneous. When cultivated in a coagulum of rat plasma, normal rat fibroblasts did not liquefy the fibrin (Fig. 2). But if sarcomatous fibroblasts were cultivated in rat plasma, they always destroyed the coagulum down to the glass after 4 or 5 days (Fig. 3). This phenomenon gave the medium an appearance similar to that of the cultures of Rous sarcoma.[11]

2. Acid Production.—It has been shown by Rous[12] that a fragment of tissue embedded in plasma rapidly modifies the adjacent medium which becomes acid to litmus. In order to ascertain whether sarcomatous fibroblasts produce more acid than normal fibroblasts, both cell types were cultivated in a flask containing a plasma coagulum and phenol red. This dye is less toxic than some other indicators, according to the findings of Rous,[13] and is well adapted to show the changes in the pH which might be expected to occur in such experiments. After the colonies had been embedded in 1 cc. of diluted chicken plasma, coagulated by 1 drop of embryo chick juice, and washed as usual, 1 cc. of Tyrode solution containing 0.04 per cent phenol red was injected into the flask. After half an hour, it was removed and replaced

[11] Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.

[12] Rous, P., *J. Exp. Med.*, 1913, xviii, 183; *Proc. Soc. Exp. Biol. and Med.*, 1911–13, ix–x, 161.

[13] Rous, P., *J. Exp. Med.*, 1925, xli, 451.

with embryo tissue juice containing 0.02 per cent phenol red. After a few hours, the sarcomatous colonies appeared golden yellow, while the normal tissues were pinkish orange. The cultures were allowed to grow for a few days. Normal and sarcomatous fibroblasts multiplied at about the same rate. Although they were in practically identical metabolic conditions, the sarcomatous tissues became bright yellow, while the normal colonies remained pinkish orange. When normal and sarcomatous tissues, instead of being cultivated as separate units, were caused to live in symbiosis, a heterologous tissue made up of patches of both cells was obtained. After the colony had been stained with phenol red, golden yellow spots appeared on a pink background. The examination of the flasks under low power showed that the yellow islands were composed of sarcomatous fibroblasts which could easily be distinguished from the normal cells by their coarser appearance. As might be expected in the light of Warburg's experiments,[14] sarcomatous fibroblasts produced much more acid than normal fibroblasts, when both cell types were placed in identical metabolic conditions. They surrounded themselves with a yellow area which extended more or less into the pink coagulum. They appeared to thrive in a pericellular fluid more acid than that about normal fibroblasts.

Optimum H Ion Concentration of the Medium.

The optimum H ion concentration of the medium was found to differ slightly for the two cell types. The sarcomatous fibroblasts must be cultivated in a well buffered medium at a pH of 7.5. The normal rat fibroblasts grow better at a pH of about 7.3 to 7.4. This slight difference is probably connected with the large quantity of acid produced by the sarcomatous fibroblasts.

Food Requirements.

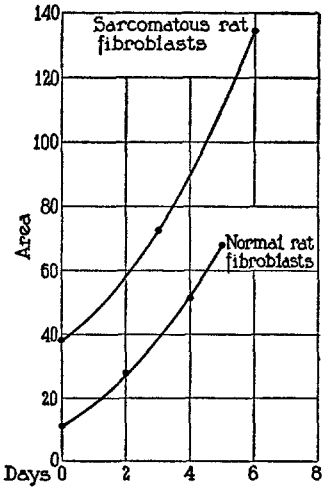
When both cell types were cultivated in chick embryo juice, they multiplied indefinitely and their rate of growth was about equal, as shown in the nine experiments summarized in Table II. The growth of the normal and sarcomatous fibroblasts of a typical experiment is expressed in Text-fig. 2. In calf liver digest, they behaved in a differ-

[14] Warburg, O., *Naturwissenschaften*, 1927, xv, 1.

TABLE II.

Effect of Tyrode Solution and Chick Embryo Juice on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Period of growth	Control in Tyrode solution	Experiment in chick embryo juice	Ratio: $\frac{E}{C}$	Period of growth	Control in Tyrode solution	Experiment in chick embryo juice	Ratio: $\frac{E}{C}$
		days				*days*			
1	6363-C					4	1.72	3.84	2.24
2	6363-C					4	1.50	2.50	1.66
3	6363-C					4	1.02	2.25	2.25
4	6425-C					5	1.25	4.03	4.03
5	6414-C	6	1.51	5.88	3.89				
6	6414-C	6	2.11	6.26	2.97				
7	6438-C	5	2.10	5.01	2.38				
8	6463-C					5	0.93	2.62	2.81
9	6463-C					5	0.90	3.42	3.80
Average.....		5.67	1.91	5.72	3.08	4.5	1.22	3.11	2.80



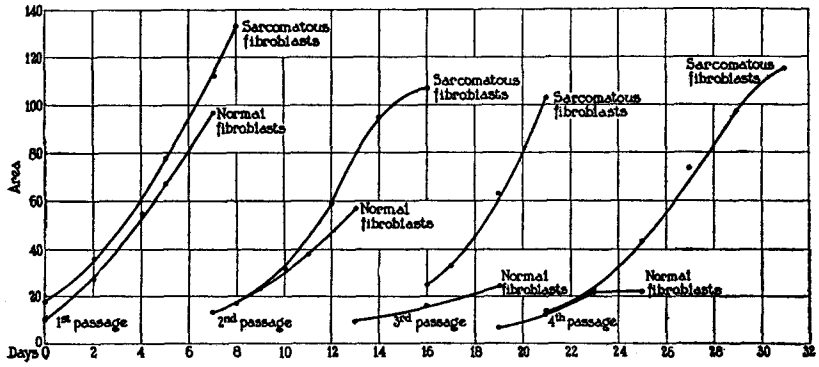
TEXT-FIG. 2. Experiments 6487-C and 9907-D. Growth of normal and sarcomatous fibroblasts of the rat in chick embryo juice.

TABLE III.

Effect of Chick Embryo Juice and Calf Liver Digest on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts [15]				Relative increase: Sarcomatous fibroblasts			
		Period of growth	Control in chick embryo juice	Experiment in calf liver digest	Ratio: $\frac{E}{C}$	Period of growth	Control in chick embryo juice	Experiment in calf liver digest	Ratio: $\frac{E}{C}$
		days				*days*			
1	6418-C	5	3.22	3.66	1.13				
2	6418-C	5	3.94	3.69	0.93				
3	6418-C	5	3.14	3.97	1.26				
4	6445-C	12	3.99	3.04	0.69				
5	6445-C	13	4.85	3.65	0.75				
6	6445-C	13	3.67	3.26	0.89				
7	6420-C					8	5.00	6.50	1.30
8	6420-C					8	4.40	6.63	1.50
9	6451-C					16	4.90	5.20	1.06
10	6451-C					16	5.35	5.14	0.97
11	6488-C	7	9.20	11.02	1.20				
12	6488-C	7	8.72	8.30	1.05				
13	6479-C					21	2.04	2.22	1.09
14	6479-C					21	1.38	3.07	1.38
15	6507-C					28	5.00	6.00	1.20
16	6505-C					31	5.64	7.00	1.24
17	6505-C					31	6.17	8.31	1.34
18	6517-C	13	3.74	3.04	0.81				
19	6538-C	19	2.68	1.10	0.45				
20	6538-C	19	4.27	1.44	0.34				
21	6553-C	23	4.38	1.92	0.44				
22	6553-C	23	5.97	1.20	0.20				

[15] The figures express the relative increase of the tissue during the last passage, and not during the entire period of growth.



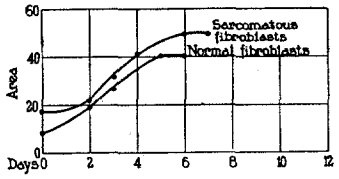
TEXT-FIG. 3. Experiments 6420-C and 6488-C. Effect of calf liver digest on normal and sarcomatous fibroblasts of the rat.

ent way. Sarcomatous fibroblasts, studied in the twenty-two experiments summarized in Table III and in Text-fig. 3, multiplied more actively in calf liver digest than normal fibroblasts did. The duration of their activity in calf liver digest was unlimited, while the normal cells did not multiply in the same medium for more than 3 or 4 weeks

TABLE IV.

Effect of Tyrode Solution and Chicken Serum on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$	Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$
		days				*days*			
1	6477-C					5	1.32	2.00	1.51
2	6478-C					7	1.00	1.52	1.52
3	6493-C					14	1.59	1.44	0.90
4	6486-C	4	2.70	3.50	1.30				
5	6486-C					4	0.80	1.24	1.55
6	6499-C	5	3.45	3.60	1.04				
7	6499-C					5	3.07	3.04	1.10
8	6512-C	10	2.44	3.22	1.32				
9	6512-C					10	1.23	1.10	0.89



TEXT-FIG. 4. Experiments 6486-C and 6499-C. Effect of chicken serum on normal and sarcomatous fibroblasts of the rat.

despite a few transfers. Calf liver digest fulfilled all the food requirements of sarcoma cells, but it failed to support the indefinite proliferation of normal rat fibroblasts.

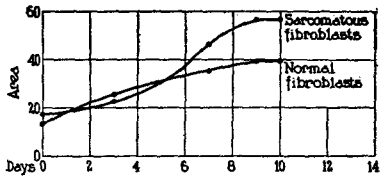
The effect of chicken serum on both cell types was ascertained in nine experiments (Table IV). In it the duration of life of both normal and sarcomatous fibroblasts did not exceed 8 days (Text-fig. 4). It is

evident that the cells do not utilize chicken serum. A similar investigation was carried out in seven experiments with rat serum (Table V). Normal and sarcomatous fibroblasts did not feed on rat serum proteins. They died after less than 7 days (Text-fig. 5.) Were it not for the observations with calf liver digest, one might suppose sarcom-

TABLE V.

Effect of Tyrode Solution and Rat Serum on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$	Duration of lite	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$
		days				*days*			
1	1513-H	5	0.95	1.03	1.08				
2	1513-H	5	2.39	1.90	0.80				
3	1513-H					5	1.40	2.00	1.42
4	1513-H					5	1.80	1.82	1.00
5	6506-C					5	1.60	2.10	1.31
6	1517-H	9	4.30	1.90	0.44				
7	1517-H					9	1.58	2.18	1.40
Average.....		6.33	2.55	1.61	0.77	6	1.60	2.03	1.28



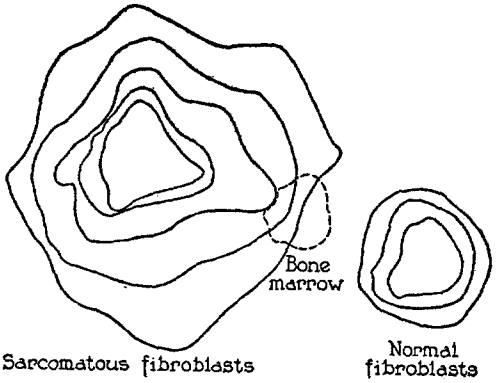
TEXT-FIG. 5. Experiment 1517-H. Effect of rat serum on normal and sarcomatous fibroblasts of the rat.

atous fibroblasts to have the same food requirements as normal fibroblasts.

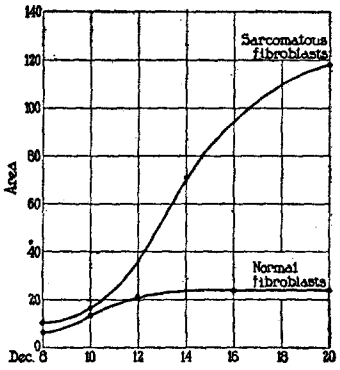
Effect of Bone Marrow on Normal and Sarcomatous Fibroblasts.

In a first series of experiments, cultures of sarcomatous and normal fibroblasts were divided into two equal parts. At an equal distance from two half cultures of malignant and normal fibroblasts, a little

fragment of rat bone marrow was placed. The medium contained diluted rat serum, and no embryonic juice. The other halves of the sarcomatous and normal colonies were cultivated as controls in a flask which contained rat serum and no bone marrow. The tracings of the tissues were made under the projectoscope and the growth of each fragment was ascertained during successive days (Text-fig. 6). The results of the four experiments of this series were constant. The ameboid cells which spread from the bone marrow multiplied more abundantly around the sarcomatous than the normal colony. The growth of the sarcomatous fibroblasts became far greater than that of



TEXT-FIG. 6.



TEXT-FIG. 7.

TEXT-FIG. 6. Experiment 1492-H. Effect of rat bone marrow on sarcomatous and normal fibroblasts of the rat in rat serum.

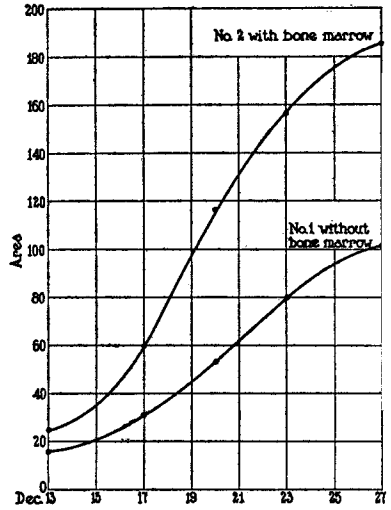
TEXT-FIG. 7. Experiment 1492-H. Effect of rat bone marrow on normal and sarcomatous fibroblasts of the rat in rat serum.

the normal (Text-figs. 6, 7). Both the controls in rat serum without bone marrow grew at equally slow rates and for only a short time. It was obvious that the multiplication of the sarcomatous fibroblasts was markedly favored by the presence of the bone marrow, while the normal fibroblasts responded but slightly. In a second series of experiments, a culture of sarcomatous fibroblasts was divided into two parts. One fragment was cultivated in rat serum without bone marrow, and the other with bone marrow. The fragment located close to the bone marrow grew faster than the isolated one (Text-fig. 8). The

sarcomatous fibroblasts apparently received from the ameboid cells of the bone marrow, directly or indirectly, certain substances which promoted their multiplication.

SUMMARY AND DISCUSSION.

It is an important fact that, after many months of life *in vitro*, in a medium composed exclusively of chicken plasma and embryonic juice or calf liver digest, the strain of fibroblasts isolated from Sarcoma 10 of the Crocker Foundation had kept its malignancy unimpaired. The indefinite persistence of this characteristic in a pure strain of



TEXT-FIG. 8. Experiment 1518-H. Effect of rat bone marrow on sarcomatous fibroblasts of the rat.

mammalian tissue renders possible, under ideally precise conditions, a study of the properties which are specific to malignancy. It opens a new era in the investigation of experimental cancer of mammals. By a method similar to that here described, Fischer and Laser have isolated the active elements of Ehrlich and Flexner-Jobling carcinomas,[6] and we have recently obtained pure cultures of the Jensen sarcomatous fibroblasts. There is little doubt that the malignant constituents of other experimental tumors can be isolated in the same

manner, and the properties associated with their unlimited growth within the body discovered.

The pure strain isolated from Sarcoma 10 is composed of fibroblasts. The cells possess all the morphological characteristics of their type. Their mode of locomotion does not differ from that of the normal fibroblasts. Their colonies are built in the same manner. However, they are larger and coarser than the normal fibroblasts of the rat when observed in pure culture (Fig. 1, *A*). Possibly, there is some other morphological difference that has not been detected by our methods of examination, such as an increase in the number of chromosomes or a change in their shape or volume. No abnormal mitoses or inclusions in the nucleus or the cytoplasm have been observed. When cultivated in a pure state, the sarcomatous fibroblasts are free from the secondary factors which *in vivo*, as well as in the mixed cultures of fresh tumor, may alter their appearance. They never degenerate and die, but multiply indefinitely. To all appearances, they are healthy cells. The malignancy that they display within the body must not be attributed to a diseased condition, but to the presence of some new physiological property.

The sarcomatous fibroblasts possess no more inherent growth energy than the normal ones. Both cell types show approximately the same residual energy in Tyrode solution and the same duration and rate of growth when placed in a nutrient medium. Like normal fibroblasts, the malignant cells multiply indefinitely in chick embryo juice, and die after a few days if cultivated in chicken or rat serum. However, they differ from the normal cells in one of their food requirements. If cultivated in a medium composed exclusively of calf liver digest, they grow in an unlimited manner, while normal fibroblasts in such a medium die within a few weeks. Although anarchical in behavior within the body, sarcomatous fibroblasts living *in vitro* require for their proliferation conditions which are almost identical with those demanded by the normal type.

However, they differ sharply from the normal fibroblasts in two aspects: they liquefy coagulated rat plasma, while normal fibroblasts do not, and they constantly produce more acid than normal fibroblasts placed under similar metabolic condition. The dissolution of the fibrin of the solid medium shows the cultures to possess the same characteris-

tics as cultures of Rous sarcoma macrophages.[11] The coagulum assumes a striking, moth eaten appearance. The increased acid production is an expression of the phenomenon studied in the well known experiments of Warburg.[14] If the liquefaction of the fibrin is due to its digestion, the malignant cells should be considered as setting free more active proteolytic enzymes than do normal ones. As proteoses and peptones are known to cause cell proliferation,[16] an increased peptic secretion, or an enhancement of the normal peptic activity by acid production, would explain the mechanism of unlimited growth. But the cells of Sarcoma 10 do not appear to hydrolyze serum proteins, or to obtain from them the required proteoses and peptones, since they do not proliferate in a serum medium. Therefore, it is difficult to understand how they multiply within the body more actively than normal fibroblasts.

Although interstitial lymph may be assumed to possess no more nutrient properties than diluted plasma, an attempt was made to ascertain whether it contains any growth-promoting substance for Sarcoma 10. A pure culture of sarcoma cells was grafted in the subcutaneous connective tissue of a rat for 24 hours, then removed, and cultivated in a flask. Its growth energy was found to have decreased considerably, and was recovered only after several days of life in chick embryo juice. But, when the culture was allowed to stay within the rat for 4 or 5 days, a small tumor appeared. Fragments of such a tumor cultivated in flasks immediately became surrounded with a crown of emigrated macrophages. It was obvious that the pure culture of sarcomatous fibroblasts, during its short stay in the rat, had attracted macrophages from the subcutaneous connective tissue. These macrophages are a normal constituent of the tumor *in vivo*. It may be supposed that the sarcomatous fibroblasts are supplied by the macrophages, directly or indirectly, with certain substances which are growth-promoting if acted upon by ferments. It is known that in normal tissues, macrophages may have such a nutritive function. Renaut thought that the lymph cells bring to fixed cells some food that they need.[17] Later, it was found that macrophages effectively

[16] Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627.
[17] Renaut, J., *Arch. anat. micr.*, 1906–07, ix, 495.

promote the proliferation of normal fibroblasts.[18] It is possible that the growth within the body of Sarcoma 10 is due to a similar phenomenon.

This hypothesis has been submitted to experimental test, and the effect of living cells on normal and sarcomatous fibroblasts cultivated in rat serum has been investigated. When a fragment of rat bone marrow was placed between two colonies of normal and malignant fibroblasts, equidistant from both, the malignant fibroblasts proliferated far more actively than the normal ones. At the same time, the wandering cells migrating from the bone marrow became more numerous in the acid area surrounding the sarcoma than in the normal tissue. It was obvious that, directly or indirectly, the wandering cells brought to the sarcomatous fibroblasts the substances required for multiplication. Instead of being the expression of a defensive reaction, the macrophages of Sarcoma 10 may supply the fibroblasts with proteins such as exist in embryo juice or with protein split products which determine their unlimited proliferation within the organism. One may suppose that the growth of Sarcoma 10 depends on the simultaneous presence of two elements: the specific malignant cells, and the nursing macrophages.

CONCLUSIONS.

1. A pure strain of fibroblasts has been isolated from Sarcoma 10 of the Crocker Foundation. After about 16 months of life *in vitro*, the malignancy of the strain is as great as that of the original tumor.

2. The strain has been compared with a strain of normal rat fibroblasts. The malignant cells are generally larger, coarser, and more refringent than normal cells. They possess all the morphological characteristics of fibroblasts. They do not show any abnormalities and never degenerate and die. They are to all appearances healthy cells. Their mode of locomotion is identical with that of normal fibroblasts. Their colonies are larger, but the architecture is similar.

3. The residual activity of both cell types, the duration of their life, and their rate of growth in a nutrient medium are almost identical.

4. The sarcomatous fibroblasts liquefy a rat plasma coagulum while

[18] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645. Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255.

normal fibroblasts do not. They turn phenol red golden yellow whereas, under the same conditions, normal cells turn it pinkish orange.

5. Sarcomatous and normal fibroblasts of the rat multiply to an unlimited degree in chick embryo juice. They live for only a short time in rat serum and chick serum. Calf liver digest will suffice for an unlimited proliferation of sarcoma fibroblasts, but fails to support the life of normal fibroblasts for very long.

6. The presence of bone marrow greatly increases the rate of growth of sarcomatous fibroblasts cultivated in rat serum, while it only slightly affects that of the normal cells. The unlimited growth of the sarcomatous tissue in animals to which it is transplanted may be attributed to the presence of macrophages, which are a normal constituent of the tumor, and possibly are a necessary factor of its growth *in vivo*.

EXPLANATION OF PLATE 4.

FIGS. 1, *A* and *B*. *A*, Culture 6827-C. Camera lucida drawing of a cell from a pure culture of fibroblasts from a normal rat. *B*, Culture 6795-C. Camera lucida drawing of a fibroblast from Sarcoma 10. Stained with 1/20,000 Janus green and 1/20,000 neutral red. The neutral red vesicles and granules are represented in gray, the fat globules by circles, and the mitochondria by lines.

FIG. 2. Culture 1482-H-1. Colonies of normal fibroblasts after 4 days in rat plasma coagulum.

FIG. 3. Culture 1482-H-2. Colony of sarcomatous fibroblasts after 4 days in rat plasma coagulum.

FIG. 1.

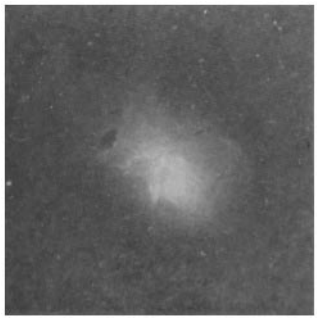


FIG. 2.

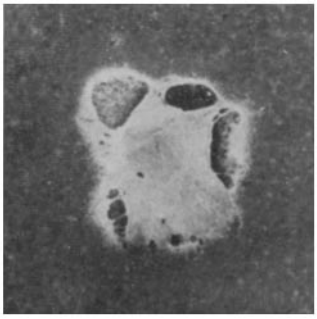


FIG. 3.

(Carrel and Ebeling: Properties of fibroblast and macrophage. III.)